Comparative Efficacy of Different Dithiocarbamates to Induce Tibial Dyschondroplasia in Poultry[1]

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ABSTRACT Tibial dyschondroplasia (TD) is a metabolic cartilage disease in poultry the natural etiology of which is not known. In the absence of biomarkers to monitor the initiation and progression of the naturally occurring disease, experimentally induced TD can provide a suitable venue to study the mechanism of its pathogenesis. Therefore, the objective of this study was to establish a streamlined experimental protocol to induce TD using dithiocarbamates and to determine a time course of its progression. Three different dithiocarbamates, dimethyldithiocarbamate, pyrrolidine dithiocarbamate, and tetramethyl thiuram disulfide (thiram), were tested with respect to their abilities to induce TD and affect different physiological factors. Our results show that chickens fed thiram during the first 2 wk of age showed a maximum TD index. Thiram appeared to be the most potent of the 3 dithiocarbamates with dimethyldithiocarbamate having the least ability to induce TD and pyrrolidine dithiocarbamate showing an intermediate potency. A transient exposure to thiram for a day or 2 was sufficient to markedly increase the incidence of TD and produce lasting damage as determined by the presence of severe lesions in a high percentage of birds at 2 to 3 wk after the treatment. Thiram affected the chondrocyte morphology of maturing zone cartilage evident by nuclear shrinkage and emptied chondrocyte lacunae during later times and also involutions of capillary vessels. Such changes were not seen in prehypertrophic zone chondrocytes of the same growth plates. Thiram reduced the BW, increased blood heterophil-to-lymphocyte ratios, and elevated serum corticosterone concentrations indicating physiological stress. However, there was no change in relative liver weights or blood clinical chemistry including the serum concentrations of Ca, P, and Cu in thiocarbamate-fed chickens. Induction of TD in young chickens by means of a short feeding protocol with thiram may be useful to study the mechanisms of pathogenesis of TD and to identify micronutrients that can provide protection against this disease.

(*Key words*: chicken, tibial dyschondroplasia, dithiocarbamate, stress, growth plate)

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INTRODUCTION

Tibial dyschondroplasia (TD) is a metabolic cartilage disease in poultry in which parts of growth plate cartilage fail to form bone, leading to the retention of a thickened plug of avascular cartilage in the proximal end of the tibia. TD is a leading cause of lameness in meat-type poultry affecting bone deformation, fracture, and bone infections that result in significant economic loss to the poultry industry and also compromise poultry welfare. Since the early description of TD by Leach and Nesheim (1965), numerous studies have characterized the pathology of TD (Edwards, 1984; Thorp et al., 1991; Leach and Lilburn, 1992; Orth and Cook, 1994; Praul et al., 2000; Farquharson, 2002). However, the natural etiology of TD has remained elusive. The cause of TD is believed to be multifactorial involving genetic, nutritional, and environmental factors (Leach and Lilburn, 1992; Orth and Cook, 1994). In the absence of suitable biomarkers to monitor the initiation and progression of the naturally occurring disease, experimentally induced disease models are often used to study the mechanism of pathogenesis in a controlled manner.

Certain dithiocarbamate fungicides, such as thiram and disulfiram, when fed to chickens, increase the incidence of TD (Vargas et al., 1983; Veltmann and Linton, 1986, Edwards, 1987; Wu et al., 1990). Dithiocarbamates are widely used as agricultural fungicides, seed fumigants, and rodent repellants, as bacteriostatic agents in many

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Abbreviation Key: DMTC = dimethyldithiocarbamate; PDTC = pyrrolidine dithiocarbamate; TD = tibial dyschondroplasia.

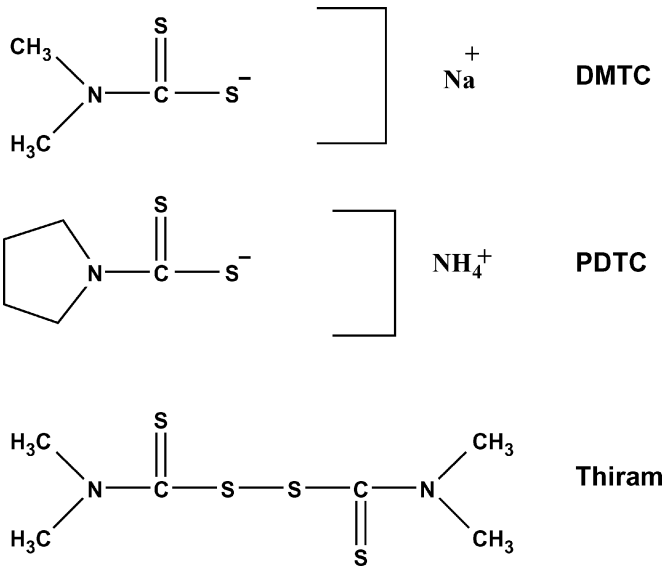

FIGURE 1. Chemical structures of different dithiocarbamates: dimethyldithiocarbamate (DMTC), pyrrolidine dithiocarbamate (PDTC), and tetramethyl thiuram disulfide (thiram).

household products, and as accelerators of vulcanization of rubber (Edwards et al., 1991; U.S. EPA, 2001). Thus, these chemicals may cause inadvertent environmental or feed contaminations resulting in leg problems in poultry (Waibel et al., 1957; Guitart et al., 1996; Wickramanayake, 1996). Because naturally occurring TD is often identified as an established lesion, it does not provide information as to the mechanism of its pathogenesis. Therefore, chemically-induced TD may provide a better insight into the disease process such as the mechanisms of the disease and secondary nutritional deficiencies that can precipitate the disease. However, studies using thiocarbamate-induced TD have been done using chronic feeding regimens and examining the lesion at the end of the study, similar to naturally occurring disease, thereby providing little information about its initiation and progression. Our objective for this study was to streamline the thiocarbamate-induced TD model so that it can be controllably used to understand the mechanisms of pathogenesis of TD. In this study we determined the effect of age on the incidence and severity of TD when chickens are exposed to TD-inducing factors such as the dithiocarbamates. We used 3 representative dithiocarbamates, dimethyldithiocarbamate, pyrrolidine dithiocarbamate, and thiram (Figure 1) based on their common chemical backbones to determine their efficacy to induce TD under identical experimental conditions. We also determined the duration of exposure to such treatments that increase the incidence of TD. Finally, we compared the influence of these dithiocarbamates on selected hematological and clinical chemistry factors, with the intent that some of these variables may provide additional insight into the pathogenesis of TD.

MATERIALS AND METHODS

Chemicals and Diets

Sodium dimethyldithiocarbamate (DMTC), tetramethyl thiuram disulfide (thiram), and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich Chemical Company.[3] Chick starter diets prepared according to NRC specifications (National Research Council, 1994) were mixed with either 50 or 100 ppm of the above chemicals as needed. Enzyme immunoassay kits for thyroxine and corticosterone were purchased from ICN Pharmaceutical Company[4] and Assay Designs Inc.,[5] respectively. All other chemicals and reagents were obtained either from Sigma-Aldrich Chemical Company[3] or as noted.

Chickens

Male broiler chicks were donated by Cobb- Vantress.[6] Chickens were raised on starter diets and water ad libitum. All applicable institutional protocols were followed for all experiments.

Age-Dependent Effect of DMTC and Thiram on TD Index

Chickens were raised on wood shavings in floor pens throughout this experiment. Except for controls, groups of 25 to 26 birds were fed diets containing 50 ppm of thiram or 100 ppm of DMTC, starting at different ages for a period of 7 d after which the chickens were necropsied to examine for the incidence and severity of TD. Thus, chickens at 3, 7, 14, 21, and 28 d of age were fed diets containing DMTC or thiram and necropsied on d 10, 14, 21, 28, and 35, respectively. Sagittal shavings of proximal tibial growth plates from bilateral sides were examined visually for the gross incidence of TD, as evident by a thickening of growth plates with opaque cartilage due to lack of vascularization and the presence of indentation some times extending into bony tibia. An arbitrary severity score was applied as follows: 0 = normal growth plate with smooth contour and off-brown tincture, 1 = mild to moderate with translucent cartilage thickened approximately to twice the size of normal, and 2 = severe with opaque white cartilage widened to span more than twice the size of a normal growth plate, indented or extending into metaphyses (Figure 2). For quantitative representation of a TD index including both incidence and severity, we used the following calculation: TD index = Σ (incidence $\times$ severity score)/number of birds). The TD index was plotted as the function of age.

Effects of PDTC

Preceding results showed DMTC to be a much less effective inducer of TD. We examined whether TD-induc-

[3]Sigma-Aldrich Chemical Company, St. Louis, MO.
[4]ICN Pharmaceutical Company, Orangeburg, NY.
[5]Assay Designs Inc., Boston, MA.
[6]Cobb-Vantress, Fayetteville, AR.

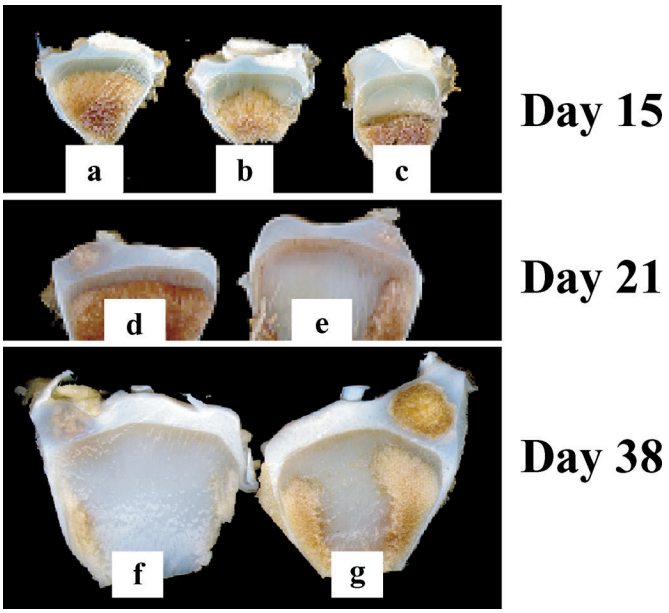

FIGURE 2. Comparative changes in the gross morphology of proximal tibial growth plate of chickens of 15 and 21 d of age treated with thiram from d 8 to 10 (upper 2 panels) and then raised on control diets for rest of the time. Lower panel: d 38 growth plates from chickens that were treated with thiram for 4 d (d 7 to 11). a and d = normal growth plates with severity score = 0; b = tibial dyschondroplasia (TD)-affected growth plate with severity score = 1; and c, e, f, and g = TD-affected growth plates with severity score = 2.

ing ability of dithiocarbamates was related to dimeric dithiocarbamates such as thiram or whether other monomeric dithiocarbamates can induce TD. We choose PDTC because it is widely used as a metabolic inhibitor and antioxidant, best known for its ability to inhibit the transcription factor NF-kB, nitric oxide synthase, and cyclooxygenase (Bessho et al., 1994). PDTC has been shown to inhibit nitric oxide production in chicken tibial-explant cultures (Orth, 2000). As per prior experimental results, we used 7-d-old broilers and fed diets containing DMTC (100 ppm), PDTC (100 ppm), or thiram (50 ppm) for 7 d,

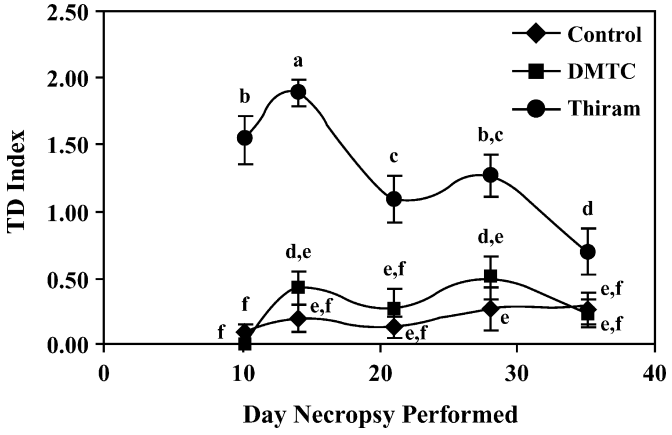

FIGURE 3. Age-dependent changes in the tibial dyschondroplasia (TD) index of broiler chickens fed with control diet or diet containing 100 ppm DMTC or 50 ppm of thiram for 7 d prior to necropsy. $^{a-e}$TD index values without a common letter differ significantly ($P \leq 0.05$).

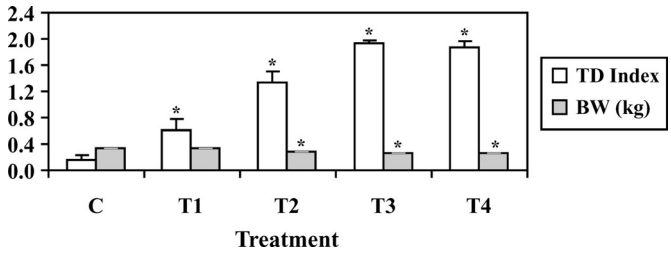

FIGURE 4. Effects of feeding duration with diet containing 50 ppm thiram on TD index and body weights of broiler chickens at age of d 14. C = control diet; T1 to T4 = fed thiram-diet for 1 to 4 d, respectively. *Significant difference ($P \leq 0.05$) between treatment and control.

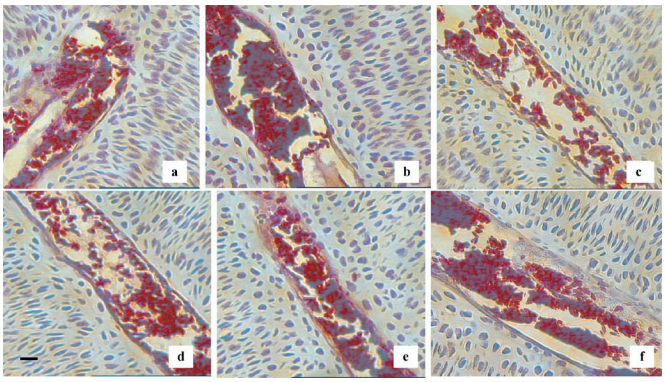

FIGURE 5. Comparative histology of proximal zone (prehypertrophic) growth plate of chickens fed control (upper panel) or 100 ppm thiram-containing diet for 2 d (lower panel) showing chondrocytes and blood capillaries. a and d: d 14 growth plate; b and e: d 21 growth plate; c and f: d 28 growth plate (100× magnification). Bar = 10 μm.

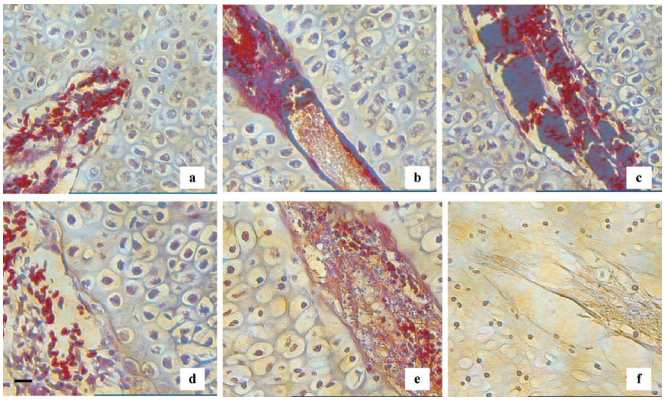

FIGURE 6. Comparative differences in histology of the distal zones of tibial growth plate of control- (upper panel) or thiram diet-fed (lower panel) chickens showing chondrocytes and blood capillaries. a and d: d 14 growth plate; b and e: d 21 growth plate; c and f: d 28 growth plate (100× magnification). Bar = 10 μm.

necropsied on d 14, and scored for the TD index. One group of birds was also treated with feed containing a DMTC concentration twice that of the 100 ppm molar equivalent of thiram.

Transient Feeding with Thiram and Its Effects

The following experiment was conducted to determine if a transient feeding between 1 and 4 d with thiram would induce TD and whether such treatments would be sufficient to cause lasting changes in growth plates in terms of showing TD lesions in the later ages. In the first experiment, 1-wk-old chickens were provided with feed containing 50 ppm thiram for 1, 2, 3, or 4 d after which the birds were returned to normal feed through d 15 when BW and TD indices of birds were evaluated. In a separate experiment 2 groups of birds were fed either a control diet or diet containing 50 ppm thiram from d 7 through 11 and then continued on a normal starter diet until 38 d of age when necropsy was performed to determine the TD indices.

Induction of TD Using a Short Feeding Protocol with Different Dithiocarbamates

A short duration feeding protocol for 48 h was attempted to determine the comparative efficacy of different thiocarbamates to induce TD and to see if their effects persisted after withdrawal of the chemicals. One-day-old broiler chicks were raised in Petersime battery cages with 12 to 13 birds per cage. On d 8 feed was withdrawn overnight for a period of approximately 12 h, after which individual treatment groups (25 chicks per group) were provided with a weighed amount of feed containing 100 ppm of DMTC, PDTC, or thiram for 48 h. Control birds were treated identically except that they were given normal feed. BW of all chickens were measured at the beginning of feed treatment and at the time of necropsy to determine percentage increase in BW. Feed consumption during the 48-h treatment was determined by calculating the feed weight at the beginning and the end of the treatment period in order to determine an estimated quantity of chemicals consumed. After 48 h, chickens in all groups were given normal feed and kept for an additional period of 24 h in the same cages before they were moved to floor pens. Birds were maintained with normal feed and water until the day of necropsy at 14, 21, and 28 d of age. As many xenobiotics can cause hepatomegaly, which is a response related to catabolic detoxification mechanisms (Wilson and Kliewer, 2002), we measured changes in relative liver weights in both control and dithiocarbamate-fed chickens to determine whether dithiocarbamates affected liver weights. The growth plates were examined for the incidence and severity of TD. Representative growth plates from 8 to 10 birds were fixed in neutral buffered formalin for histology.

Hematological and Clinical Chemistry Changes

In some studies we also determined changes in selected hematological and blood chemistry factors to determine whether these variables would provide possible insight to the pathogenesis of TD. In these experiments, blood was collected by cardiac puncture from 11 to 12 birds per group prior to necropsy. Potassium-EDTA anti-coagulated blood was used for hematological profiling and serum for clinical chemistry. Hematological evaluation was carried out with a CellDyn blood cell counter.[7] Selected serum chemistry profiles which included calcium, phosphorus, alkaline phosphatase, creatine kinase, protein, glucose, cholesterol, triglyceride, alanine aminotransferase, and aspartate aminotransferase, were determined using a Corning clinical chemistry analyzer.[8] Copper and zinc levels in serum were measured with an inductively coupled plasma spectrometer[9] after hydrolyzing the serum with 10 *N* nitric acid (1:1) for 2 h at 60°C followed by 2 h at 120°C, and diluting it with water to bring the concentration of nitric acid to 1 *N*. The concentrations of Cu and Zn were calculated using appropriately prepared standards.

Because dithiocarbamates can induce thyroid deficiency (Edwards et al., 1991), and because thyroxine is an important regulator of chondrocyte maturation (Ballock and Reddi, 1994), we measured the levels of thyroxine in select serum samples using a commercial kit.[4] Similarly, stress-associated changes in serum corticosterone were determined using a corticosterone enzyme immunoassay kit.[3]

Morphology and Histology of Growth Plate

Sagittal sections of proximal tibial growth plates were fixed with neutral buffered formalin and scanned using a Hewlett-Packard desk top scanner for gross morphology. Also, the formalin-fixed growth plates were processed for histology without any decalcification, and 5 μm thick paraffin sections were stained with hematoxylin and eosin.

Statistics

The quantitative data were evaluated using the general linear model procedure or Student's *t*-test where applicable (SAS Institute, 1988). The results were presented as mean ± SEM. A $P < 0.05$ was considered significant.

RESULTS

Age-Dependent Effect

For chicks fed the compounds for 1 wk starting on d 3 posthatch or later, an age-dependent change in the TD

[7]Abbott Laboratories, Chicago, IL.
[8]Chiron Corporation, San Jose, CA.
[9]Spectro Analytical Instruments, Fitchburg, MA.

TABLE 1. Body weight, liver weight percentage, tibial dyschondroplasia (TD) score, and representative hematological and clinical chemistry factors ± SEM of 14-d-old broiler chicks fed with control or 50 ppm thiram-supplemented diets for 4 d from d 7 posthatch through d 11 (n = 11)

Variables	Control	Thiram
BW (kg)	0.43 ± 0.02	0.35 ± 0.01*
Liver weight (%)	3.2 ± 0.1	3.1 ± 0.1
TD index	0.02 ± 0.01	1.20 ± 0.02*
White blood cells ($10^3/\mu$L)	23.4 ± 2.7	13.6 ± 1.9*
Red blood cells ($10^6/\mu$L)	1.9 ± 0.05	2.2 ± 0.09
Heterophil (%)	5.7 ± 0.8	26.4 ± 3.8*
Lymphocyte (%)	88.2 ± 1.5	62.1 ± 5.1*
Heterophil-to-lymphocyte ratio	0.07 ± 0.01	0.52 ± 0.11*
Monocyte (%)	4.7 ± 1.1	8.4 ± 1.7*
Hematocrit (%)	27.9 ± 0.7	31.9 ± 1.3
Platelet (k/μL)	4.1 ± 0.5	4.0 ± 0.7
Ca (mg/dL)	10.8 ± 0.1	10.3 ± 0.1
P (mg/dL)	5.7 ± 0.3	5.7 ± 0.2
Cu (ng/mL)	0.063 ± 0.004	0.070 ± 0.005
Alkaline phosphatase (U/L)	3,523 ± 702	6,937 ± 1,570*
Creatine kinase (U/L)	6,648 ± 682	6,776 ± 1,088
Triglyceride (mg/dL)	24.0 ± 4.5	18.2 ± 1.1
Thyroxine (μg/dL)	8.1 ± 1.6	10.5 ± 1.0
Corticosterone (ng/mL)[1]	6.3 ± 1.4	12.2 ± 1.8*

*Differences between the indicated values in the control and Thiram columns were significant, $P \leq 0.05$.
[1]n = 6.

index was apparent in response to thiram; d 3 and 7 thiram-fed chicks showed the highest TD indices (Figure 3). However, under similar conditions, DMTC did not induce any TD, and the TD index was not substantially different from controls.

PDTC Effect

When the TD index of chickens fed 100 ppm of PDTC was compared with that of control or 100 ppm DMTC-fed chickens, the PDTC group showed a higher TD index but the index was much lower compared to 50 ppm thiram-fed birds (control: 0.07 ± 0.03; DMTC: 0.12 ± 0.07; PDTC: 0.95 ± 0.17, $P < 0.05$; thiram: 1.70 ± 0.15, $P < 0.05$). Increasing the concentration of DMTC to twice the 100 ppm molar equivalent of thiram did not increase the TD index (0.03 ± 0.02). When compared with the control group (0.38 ± 0.01 kg), PDTC did not impair growth as reflected in similar BW (0.37 ± 0.01 kg) whereas both DMTC and thiram reduced BW (0.33 ± 0.02 kg, 0.32 ± 0.01 kg, $P < 0.05$, respectively).

Transient Feeding with Thiram and Its Subsequent Effects

As little as 1-d of treatment with thiram was sufficient to raise the TD index of 1-wk-old chickens compared with controls. There were no statistical differences in the TD indices of chickens fed for 3 or 4 d with thiram. Also, thiram feeding caused a decrease in BW, which was further reduced by the longer feeding schedule (Figure 4). The long-term effect of 4 d of thiram feeding on the TD index of 38-d-old chickens was significantly higher compared with control-fed birds; most thiram-fed chickens showed severe lesions extending deep into the metaphyses (control: 0.48 ± 0.13; thiram: 1.58 ± 0.20; Figure 2, f and g). The BW of thiram-fed birds was lower, blood levels of heterophils were higher, and white blood cell counts were lower (Table 1).

Induction of TD Using a Short Treatment Protocol and Its Effects

Using the short feeding protocol for 48 h, thiram caused a dramatic increase in the TD index compared to other dithiocarbamates (Table 2). The TD lesions were larger in size and severity in 21- and 28-d-old birds (Figure 2e, f, and g), in some cases spanning the entire growth plate. The histology of proximal growth plate (prehypertrophic zone) from thiram-treated chickens was not much different from that of control-fed birds on 15, 21, or 28 d of age (Figure 5). However, there were evolving changes in the distal growth plate histology of chickens that were fed thiram. In 15-d-old chickens, the histological differences between control and thiram-fed birds were not as obvious as in 21- or 28-d-old birds. In both the later groups, the chondrocyte nuclei were pyknotic and the chondrocyte size appeared reduced by d 28. The effects were progressive with many chondrocytes from 28-d-old chickens showing nucleus-denuded cellular lacunae only (Figure 6d, e, and f). Comparatively, chondrocyte nuclei from 28-d-old birds also appeared smaller than in 21-d-old growth plate chondrocytes. Additionally, the blood vessels from these areas showed dramatic differences with progressive atrophy of capillaries and loss of hematopoeitic cellularity (Figure 6e and f). The DMTC- or thiram-treated birds showed a consistent decrease in BW at all times compared with controls (Tables 1 and 2). However, these treatments did not change the relative liver weights.

TABLE 2. Changes in BW and tibial dyschondroplasia (TD) index (± SEM) of chickens on different days after 48 h of feeding with different dithiocarbamates (100 ppm in feed, n = 25 birds per group)

Parameter	Age (d) at necropsy	Control	DMTC[2]	PDTC	Thiram
Increase in BW[1] (%)	15	197 ± 5[a]	178 ± 5[b]	197 ± 4[a]	156 ± 4[c]
	21	575 ± 11[a]	535 ± 12[b]	558 ± 14[a]	505 ± 19[b]
	28	1,078 ± 32[a]	ND	ND	1,026 ± 6[b]
TD index	15	0.08 ± 0.06[b]	0.13 ± 0.07[b]	0.21 ± 0.09[b]	1.92 ± 0.06[a]
	21	0.23 ± 0.09[b]	0.33 ± 0.12[b]	0.39 ± 0.13[b]	1.70 ± 0.14[a]
	28	0.29 ± 0.14[b]	ND	ND	1.73 ± 0.03[a]

[a–c]Values with no common superscript in a row differ significantly ($P \leq 0.05$).

[1]Percentage increase in BW was calculated relative to BW on d 7 when the feed was withdrawn.

[2]DMTC = dimethyldithiocarbamate; PDTC = pyrrolidine dithiocarbamate; ND = not determined.

Hematological and Clinical Chemistry Changes

There were no differences in the serum Ca, P, or creatine kinase levels but the alkaline phosphatase level showed an elevation in thiram-fed chickens (Table 1). Neither copper nor zinc content of serum was affected by any treatment. There was no negative effect of thiram on the serum thyroxine concentration. On the other hand, the serum corticosterone level of thiram-fed birds was increased by approximately 2-fold (Table 1). A similar trend was also evident in birds that were treated for only 2 d with thiram. There was a consistent decrease in white blood cell counts in both PDTC- and thiram-fed chickens on d 21 (Table 3). The heterophil levels were elevated with a concurrent decrease in the levels of lymphocytes, culminating in an overall increase in the heterohil-to-lymphocyte ratios in dithiocarbamate-treated chickens (Table 3).

Essentially, there were no differences in the conventional clinical chemistry profiles between control and dithiocarbamate-treated birds (not shown). By d 28, the hematological profiles of control and thiram-fed birds were comparable (Table 3). However, the thiram-fed chickens showed an increase in serum concentration of corticosterone on d 15.

DISCUSSION

The ability of thiram and its tetraethyl analogue, disulfiram, to induce leg problems and TD in poultry has been known for many years (Waibel et al., 1957, Vargas et al., 1983; Edwards, 1987; Wu et al., 1990; Guitart et al., 1996; Wickramanayake, 1996). In the past, studies with thiram-induction of TD have been carried out using chronic feeding regimens and examining for the presence of the lesions at the termination of the experiments (Veltmann and Linton, 1986; Edwards, 1987). However, the objectives of our study were to determine whether there was an age-dependent sensitivity to dithiocarbamates during which chickens may be more susceptible to the induction of TD, and to determine what minimum duration of exposure to such an inducer is needed for the pathogenesis of TD. Finally, we were interested in developing an experimental protocol that can be used to study the mechanisms of the induction and the progression of TD. Results show (1) 1-

TABLE 3. Selected hematological and clinical chemistry changes (± SEM) at different ages following a short 2-d treatment with different dithiocarbamates (n = 12 birds per group)

Variable	Age (d)	Control	DMTC[2]	PDTC	Thiram
White blood cells cells ($10^3/\mu L$)	15	18.7 ± 2.1[a]	17.5 ± 2.4[a]	12.5 ± 2.0[a]	12.6 ± 2.4[a]
	21	15.7 ± 2.7[a]	13.5 ± 2.0[a,b]	9.5 ± 1.5[b]	8.8 ± 1.7[b]
	28	15.9 ± 2.8[a]	ND	ND	16.6 ± 3.2[a]
Heterophil (%)	15	6.1 ± 1.8[b]	9.3 ± 2.7[b]	9.5 ± 3.7[b]	20.3 ± 3.6[a]
	21	18.8 ± 3.6[b]	21.6 ± 5.0[b]	30.1 ± 4.4[a,b]	40.4 ± 2.88[a]
	28	31.6 ± 5.3	ND	ND	28.8 ± 5.0
Lymphocyte (%)	15	83.9 ± 2.1[a]	81.0 ± 3.8[a]	73.0 ± 5.9[a,b]	65.8 ± 4.2[b]
	21	68.8 ± 7.01[a]	65.4 ± 6.2[a]	57.5 ± 6.3[a]	36.6 ± 5.4[b]
	28	57.5 ± 6.9	ND	ND	59.3 ± 7.5
Heterophil-to-lymphocyte ratio	15	0.08 ± 0.02[b]	0.15 ± 0.06[a,b]	0.19 ± 0.11[a,b]	0.36 ± 0.09[a]
	21	0.38 ± 0.11[b]	0.48 ± 0.17[b]	0.68 ± 0.19[b]	1.49 ± 0.30[a]
	28	0.87 ± 0.29	ND	ND	0.94 ± 0.42
Corticosterone[1] (ng/mL)	14	6.51 ± 1.37[b]	4.84 ± 1.12[b]	8.94 ± 1.88[a,b]	12.02 ± 1.97[a]

[a,b]Values with no common superscript in a row differ significantly ($P \leq 0.05$).

[1]n = 7 birds per group.

[2]DMTC = dimethyldithiocarbamate; PDTC = pyrrolidine dithiocarbamate; ND = not determined.

wk-old broiler chicks were most prone to the TD-inducing effects of thiram, (2) a transient exposure to thiram for as little as 1 d was sufficient to increase the TD index, and (3) thiram appeared to differentially affect hypertrophic zone cartilage and blood vessels as compared with prehypertrophic zone cartilage and the blood vessels therein.

The age dependence of the susceptibility of chickens to thiram suggests that the heightened vulnerability to thiram or perhaps other such TD-inducing agents depends upon the growth rate of chickens which is fastest during the first 2 to 3 wk of age (National Research Council, 1994). However, the birds remained susceptible to the induction of TD during all times of our study. Comparison among 3 dithiocarbamates showed thiram to be the most potent inducer of TD. PDTC, which is widely used as a metabolic inhibitor (Schreck et al., 1992; Zeigler-Heitbrock et al., 1993; Bessho et al., 1994), was intermediately effective using a chronic feeding regimen for 7 d but was ineffective using a short protocol of 2 d of treatment. It has been suggested that PDTC undergoes metabolic conversion to thiram (Burkitt et al., 1995; Nobel et al., 1995; Elskens and Penninckx, 1997; Wild and Mulcahy, 1999) which may be why it can induce TD. It is not clear, however, whether thiram by itself or its metabolites are responsible for the pathogenesis of TD. The toxicological effects of dithiocabamates are generally attributed to their metabolites, of which carbon disulfide is the most prominent and causes axon neuropathy (US EPA, 2001). Metabolically, thiram has to be converted to DMTC for its further degradation to several smaller metabolites including carbon disulfide (Edwards et al., 1991; Johnson et al., 1996). Because the monomeric DMTC failed to induce any significant incidence of TD, we conclude that the TD-inducing efficacy is unlikely to be mediated by metabolites other than thiram itself. Additionally, the results of the studies on long-term effects of transient treatment with thiram suggests that the chondrocytes that are affected by thiram apparently are not readily removed and thus accumulate contributing to the severity of TD lesions.

The histology of TD-affected areas of growth plates of chickens transiently fed for 2 d with thiram showed interesting differences in chondrocyte morphology and blood capillaries. While the chondrocytes from TD-affected zones of 15-d-old thiram-treated birds did not appear much different from those of the controls, they were different in 21- and 28-d-old chickens. The nuclei of many chondrocytes in the distal zones of growth plate were pyknotic and shrunken in size by d 21; chondrocytes from lesions in 28 d old-birds showed further shrinkage and emptied chondrocyte lacunae, some of the hallmark features of TD lesions (Riddell, 1981, Hargest et al., 1985; Thorp et al., 1991, Leach and Lilburn, 1992). This is also consistent with the biochemical observation that the DNA contents of TD-affected growth plate cartilages were much lower than that of normal growth plate cartilage possibly because of degradative loss of nucleic acids due to DNA fragmentation (Freedman et al., 1985; Rath et al., 1996; Praul et al., 1997; Rath et al., 1998). It appears as if the cytoplasmic tethers that hold the nuclei are gradually lost toward the terminal phase of the disease culminating in the loss of nuclei from the cartilage lacunae. The hypertrophic zone capillaries also showed extensive involution changes from d 15 through d 28 with capillaries having regular cellular components at the early time period, capillary canals containing dying cells at d 21, and degenerating blood vessels on d 28. On the other hand, prehypertrophic zone chondrocytes and blood vessels therein showed little changes during these periods.

The anti-angiogenic effect of thiram has been observed using endothelial cell culture and in rapidly growing tumor tissues (Marilkovsky, 2002). In previous studies with naturally occurring TD lesions in turkeys, we have shown extensive capillary death (Rath et al., 1998). However, the differential effect of thiram on hypertrophic zones vs. the prehypertrophic zones of the same growth plate is intriguing. Perhaps it leads to the accumulation of a dead plug of cartilage after the thiram treatment is discontinued. The transition zone chondrocytes undergo significant volume changes during hypertrophy which can be as much as 5 to 10 times their original volume (White and Wallis, 2001). We surmise that during this development chondrocytes experience rapid changes in membrane fluidity which may allow incorporation of lipophilic compounds such as thiram into those cells initiating cytotoxic effects. The cytotoxic effects of thiram can arise from its inhibitory effects on glutathione metabolism, membrane damage, genetic activation of death signals, or selective inactivation of mechanisms that lead to cartilage remodeling and osteogenesis (Rath et al., 1994; Nobel et al., 1995; Balakirev and Zimmer, 2001; Cereser et al., 2001). Cartilage is relatively isolated in regard to a ready removal of dead or damaged cells through phagocytosis or through vascular channels, particularly when blood capillaries are affected. This could mean that much of the metabolic degradation of thiram or its disposal from the tissues may be impaired leaving the chemical relatively intact to initiate further damage to successive generations of transitional chondrocytes at the vicinity of the dead cells until the chemical gradient is sufficiently diluted to carry out no further damage. However, these mechanisms are speculative and need further experimental confirmation. It should also be noted that although both DMTC and PDTC are aqueous-soluble, thiram is hydrophobic, which may facilitate its accumulation in the fat depot and incorporation into phospholipid membranes.

Rapid growth is considered to be a predisposing factor for TD in meat-type poultry. Reducing BW with feed restriction as well as by supplemental 1,25-dihydroxycholecalciferol is known to reduce the incidence of TD (Edwards, 1989; Xu et al., 1997; Edwards, 2000). However, the relation between growth and the incidence of TD has been questioned (Leach and Lilburn, 1992, Praul et al., 2000). In the current study chickens with induced TD showed a consistent reduction in BW indicating that the pathogenesis of TD may be independent of the BW of birds, at least, under present experimental conditions. Besides inducing TD, thiram also had a profound effect on several hematological factors that were indicative of

stress. Thiram consistently increased the blood levels of heterophils, and the heterophil-to-lymphocyte ratio which is indicative of stress (Gross and Siegel, 1986). Additionally, the blood levels of corticosterone were elevated in the thiram-fed but not in DMTC-fed birds. Because a higher incidence of TD has been linked to many adverse environmental conditions such as extreme weather, crowding, and poor floor pen conditions (Grashorn, 1992; Leach and Lilburn, 1992; Orth and Cook, 1994; Sanotra et al., 2001), it is likely that physiological stress could be contributory or at least a risk factor in the pathogenesis of TD. However, this issue has not been addressed in the literature. Dithiocarbamates have also been shown to have anti-thyroid effects (Edwards et al., 1991; U.S. EPA, 2001), and since thyroxine is an important regulator of chondrocyte maturation (Ballock and Reddi, 1994), a thiram-induced deficiency of thyroxine could potentially contribute to the failure of the hypertrophic chondrocytes. However, our results with 7 d of treatment with thiram did not show any decrease in blood thyroxine levels; on the contrary, a numerical increase in the serum thyroxine level was noted in the thiram-fed birds. Although lipophilic xenobiotics are likely to induce hepatomegaly because of the involvement of liver in the metabolic degradation of these chemicals (Wilson and Kliewer, 2002), there were no changes in relative liver weights, indicating the absence of hepatotoxicity under these treatments. Neither were there any changes in the serum levels of alanine or aspartate aminotransferases, which would indicate liver toxicity. The dithiocarbamates including thiram had no effect on blood Ca, P, Fe, Zn, or Cu levels. Unlike its effects on hematological factors, thiram had very little effect on blood chemistry.

While the mechanism of action of thiram to induce TD remains to be understood, our results show that a short exposure of growing birds to certain dithiocarbamates may be enough to induce a substantial incidence and severity of TD. With the short feeding protocol to induce TD by thiram it may be possible to study the mechanisms of induction and the progression of this disease in a controlled manner. It may also be possible to screen for the protective effects of different micronutrients against the pathogenesis of tibial dyschondroplasia using this model.

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